TITLE PAGE

Final Technical Report

PERIOD COVERED

1 December 1962 - 1 September 1963

PRINCIPAL INVESTIGATOR

Robert Charles Wood, Ph. D.

INSTITUTION

George Washington University School of Medicine, Washington 5, D. C.

SUBJECT OF THE REPORT

Studies on the Genetics of Virulence in Enteric Bacteria

CONTRACT NUMBER

DA-49-193-MD-2376

DDC AVAILABILITY STATEMENT

Qualified Investigators May Obtain Copies of This Report From DDC

SECURITY CLASSIFICATION

Unclassified

ABSTRACT

Preparing Institution:	George Washington University School of Medicine Washington,5, D.C.
Title of Report:	Studies on the Genetics of Virulence in Enteric Bacteria
Principal Investigator:	Robert Charles Wood, Ph.D.
Number of Pages, Illustrations and Date:	10,pages, 5 tables 1 September 1963
Contract Number:	DA-49-193-MD-2376
Supported by:	U. S. Army Medical Research and Development Command Department of the Army Washington 25, D.C.
Note:	Copies of this report are filed with the Defense Documentation Center, Building 5, Cameron Station, Alexandria, Virginia, and may be obtained from that agency by qualified investigators working under government contract.

Alterations were observed in the virulence of Shigella flexneri 2a for pretreated guinea pigs following hybridization by Escherichia coli K-12 Hfr strains. Genetic studies indicated gross chromosomal homology between these two species. However, recombination frequencies were lower than those obtained in comparable E. coli X E. coli crosses, and extensive transfer of the Escherichia genome was detected only occasionally. Virulence tests on Shigella hybrids carrying overlapping segments of the Escherichia chromosome revealed only one region, located between the rha$^+$ and xyl$^+$ genes, which was essential for virulence. Hybrids carrying the xyl$^+$-rha$^+$ Escherichia region as a prominent exogenote displayed intermediate virulence; haploid segregants which lost this exogenote were fully virulent, while one haploid which had incorporated it proved to be avirulent. Preliminary tests on a mouse-virulent Salmonella typhimurium hybridized by an avirulent Salmonella ebony donor indicated that, in this species also, virulence

may be lost on substitution of the *xyl*$^+$-*rha*$^+$ region. *Salmonella typhosa* strain 643WS^r, hybridized by mating with an *E. coli* Hfr strain, was found to exhibit enhanced recipient ability on remating with *E. coli* donors whose lead chromosomal region matched the genetic segment integrated by the hybrid.

I. Alterations in the virulence of Escherichia-Shigella hybrid for the guinea pig.

1. During the past year this investigation has focused on the genetic basis of virulence in enteric bacteria. One important finding has been the loss of guinea pig virulence in the 2a strain of Shigella flexneri after specific substitution of Escherichia coli genetic material, as a result of mating with E. coli K-12 Hfr strains.

2. Genetic recombination between Hfr strains of Escherichia coli K-12 and members of the Shigella group was initially demonstrated by Luria and Burrous (1957). Extensive, though incomplete, genetic homology was found to exist between these two genera. Formal et al.(1958, 1959) demonstrated a fatal enteric infection in either starved or carbon tetrachloride pretreated guinea pigs challenged orally with S. flexneri 2a. This infection produced lesions in both the large and small bowel which resembled those seen in human cases of shigellosis. The availability of both a genetic transfer system and an infection model thus enabled examination of alterations in the virulence of S. flexneri 2a for pretreated guinea pigs, following conjugation with E. coli K-12.

3. Three E. coli K-12 Hfr strains were employed in the genetic crosses: W1895, which transfers its chromosomal markers in the order origin, lactose utilization (lac$^+$), arabinose utilization (ara$^+$), rhamnose utilization (rha$^+$), indol production (ind$^+$), xylose utilization (xyl$^+$), maltose utilization (mal$^+$), fucose utilization (fuc$^+$), nicotinic acid synthesis (nic$^+$), ... galactose utilization (gal$^+$); AB 313 (origin, ind$^+$, xyl$^+$, mal$^+$, ... rha$^+$); 1362

(origin, mal$^+$, xyl$^+$, ind$^+$, ...fuc$^+$). Matings between these E. coli donors and the 2a S. flexneri strain indicated that a gross homology exists between the chromosomes of these two species. However, recombination frequencies were lower than those obtained in comparable E. coli X E. coli crosses (Table 1). Further, the predominant Shigella hybrid class acquired only the selected genetic marker from the E. coli parent; extensive transfer of the Escherichia genome was detected only occasionally (Table 2).

4. A definite progressive relation between dosage and response is not always apparent in the oral administration of Shigella to starved or carbon tetrachloride treated guinea pigs (Formal et al., 1958, 1959). Thus it was not possible to employ the LD_{50} dose as an estimate of virulence. However, it was observed that a dose of 5×10^7 to 1×10^8 Shigella organisms administered orally, under our experimental conditions, consistently killed a large proportion of animals. Assessment of virulence of parental and hybrid strains, therefore, was dependent primarily on an "all or none" type of response.

5. By testing Shigella hybrids carrying overlapping segments of the Escherichia genome, it was hoped that the minimum number of chromosomal regions responsible for the virulence of Shigella for the pretreated guinea pig might be identified. Our virulence data is summarized in Table 3. Hybrids having received lac$^+$, lac$^+$- arg$^+$, rha$^+$ and mal$^+$ from the E. coli parent were as virulent as the Shigella parental strain. Those hybrids which carried rha$^+$-ind$^+$ were uniformaly avirulent, while xyl$^+$ and xyl$^+$ - mal$^+$ hybrids were either completely virulent or avirulent in about equal proportions. Although one fuc$^+$ hybrid was virulent, the remaining fuc$^+$ hybrids tested, as well as all of the nic$^+$, fuc$^+$ - nic$^+$, and fuc$^+$ - nic$^+$ - lac$^+$ were of intermediate virulence, i.e., they consistently killed a low but significant proportion of animals. In addition, hybrids which had received the pili antigen from E. coli also displayed this intermediate virulence.

6. Examination of hybrids possessing the characteristics of segregating partial diploids strongly indicated that the observed alterations in virulence were due to the transferred Escherichia material. Several rha$^+$ - ind$^+$ - xyl$^+$ - mal$^+$ hybrids, in spite of repeated purification, segregated clones (about 1 in 10^4 cells) which exhibited the parental Shigella phenotype. When tested in the guinea pig (Table 4), these partial diploids were found to be of intermediate virulence, while their haploid segregants, which had completely lost the injected Escherichia genes, were indistinguishable from the parental Shigella strain in their virulence. On oneoccasion a haploid segregant was isolated in which the previously exogenotic fragment had become integrated. This segregant proved to be avirulent.

II. Alterations in the virulence of Salmonella - Salmonella hybrids for the mouse.

1. The indication of a genetic locus, or loci, controlling the virulence of Shigella for the pretreated guinea pig has led us to investigate the possible existence of a similar factor, or factors, in Salmonella. Hybridization of Salmonella by E. coli was initially reported by Baron, Carey and Spilman (1958). Subsequently, it has been found possible to produce Salmonella possessing donor ability, either by hybridization for a terminal E. coli Hfr marker (which often results in integration of the sex factor, F), or by infection with F, followed by selection for Hfr mutants. The use of such donors in crosses with Salmonella typhimurium, a natural pathogen for the mouse, presents an excellent system for the genetic study of virulence.

2. In preliminary experiments, S. typhimurium strain C-5, genetically marked and virulent for the mouse, was mated with an avirulent S. abony donor strain, and several hybrid classes from this mating were tested for mouse virulence. The initial findings indicate that virulence may be lost in the hybrids when substitution occurs in the xyl^+ - rha^+ region. The correspondence of this preliminary data with that obtained from the experiments with the Shigella hybrids is most encouraging. However, further studies will be necessary to confirm these findings, and to establish whether other chromosomal regions might also be involved in the virulence of S. typhimurium for the mouse.

III. Behavior of E. coli - S. typhosa hybrids on remating with E. coli Hfr strains.

1. From the outset of this investigation our hope has been to transfer the property of virulence from a virulent genetic donor to an avirulent genetic recipient. As yet, this has not been accomplished, owing to the difficulty of obtaining virulent donor strains. Salmonella, either virulent or avirulent, generally are poor recipients in genetic crosses with E. coli K-12 donors. Transfer of Hfr donor ability to virulent Salmonella strains in these cases is very hard to achieve. However, our studies on the behavior of S. typhosa hybrids produced by mating with E. coli have pointed out a method by which the genetic recipient ability of Salmonella may be increased.

2. It has been previously observed that S. typhimurium hybrids from genetic crosses with E. coli Hfr strains exhibit increased fertility when remated with the E. coli parent. This has been shown to represent selection of pre-existing, high frequency recipients from an otherwise sterile population by the first round of mating. However, upon examination of this phenomenon with S. typhosa as the genetic recipient, we discovered that, in this species, selec-

tion of high frequency recipients was not involved. Our studies showed, in fact, that the increased recipient ability of the S. typhosa hybrids was due to the presence of integrated E. coli genetic material.

3. S. typhosa strain 643WS^R was mated with the E. coli Hfrr W1895 (origin, lac$^+$, ara$^+$, . . . gal$^+$) with selection for the lead marker lac$^+$. Lactose positive S. typhosa hybrids, when remated with W1895 for the more distal marker ara$^+$, showed frequency increases of 200 times the normal frequency for transfer of this gene to previously unmated S. typhosa. It was noted that many lac$^+$ S. typhosa hybrids were heterogenotes which continually segregated lactose negative (lac$^-$) clones. When a number of these lac$^-$ segregants, which had lost the E. coli genetic material, were remated with the E. coli parent, none showed any increase in recipient ability. This provided the initial evidence that the fertility increases of the hybrids occurred as a consequence of the transferred E. coli genetic material.

4. E. coli Hfr strains W1895 and Hayes (transferring origin, ara$^+$, lac$^+$, . . . rha$^+$) were crossed with S. typhosa 643 WS^R, with selection for the single markers lac$^+$ and ara$^+$. Four classes of 643WS^R hybrids were obtained which were labelled as follows: those receiving lac$^+$ alone or ara$^+$ alone from W1895 were designated WS^R lac$^+$(95) and WS^R ara$^+$(95), respectively; those receiving these individual markers from Hayes were labelled WS^R lac$^+$(H) and WS^R ara$^+$(H). These four hybrid classes were then remated with each of the two E. coli Hfr strains in a series of eight reciprocal genetic crosses. These crosses, shown in Table 5, demonstrated that each hybrid class was capable of exhibiting significant increases in recombination frequency wherever the leading chromosomal region of

the E. coli Hfr used for remating matched the E. coli genetic segment previously integrated by the hybrid. When the alternate Hfr (which did not inject its chromosome into the region of artificial genetic homology) was employed, no increase in recombination frequency was observed. An exception was noted in the Hayes X WS^R lac^+(H) matings (Table 5, cross 6), where a 13-fold increase occurred when the E. coli segment integrated by the hybrid does not appear to match the lead chromosomal region of Hayes. Probably, these hybrids have integrated E. coli material nearer to the Hayes lead region than the absence of the ara^+ marker would indicate.

5. Thus, it is possible to increase significantly the recipient ability of S. typhosa in crosses with E. coli Hfr strains by transfer and integration of strategically located E. coli chromosomal segments. The studies of Zinder (1960) and Falkow, Rownd and Baron (1962) have shown that the homology which exists between Escherichia and Salmonella is incomplete. Presumably, then, prior establishment in S. typhosa of an Escherichia genetic segment homologous with the leading chromosomal section of the E. coli donor facilitates early genetic pairing and integration of the transferred material. Removal of the initial barrier to integration of lead Hfr markers would increase the chances of integration of more distal markers, thus accounting for the observed increase in recombination frequencies.

Summary

1. Three E. coli K-12 Hfr donors were employed in genetic crosses with the 2a strain of Shigella flexneri. The results of these matings confirmed the existence of a gross homology between the chromosomes of these two organisms.

However, recombination frequencies were lower than those obtained in comparable E. coli X E. coli crosses, and extensive transfer of the Escherichia genome was detected only occasionally.

2. Shigella hybrids carrying overlapping segments of the Escherichia genome were compared with the parental Shigella strain with regard to their virulence for starved or carbon tetrachloride treated guinea pigs. Hybrids which received lac^+, lac^+ - ara^+, rha^+, and mal^+ from the E. coli parent were as virulent as the parent strain. Hybrids carrying rha^+ - ind^+ were uniformly avirulent, while xyl^+ and xyl^+ - mal^+ hybrids were either completely virulent or avirulent in about equal proportions. With one exception, fuc^+, nic^+, fuc^+ - nic^+, and fuc^+ - nic^+ - lac^+ hybrids, as well as those receiving the pili antigen from E. coli, were of intermediate virulence.

3. Shigella rha^+ - ind^+ - xyl^+ - mal^+ hybrids, possessing the characteristics of segregating heterogenotes, were found to be of intermediate virulence, while their haploid segregants, which had lost the E. coli genetic material, were fully virulent. One haploid segregant, which had integrated the previously exogenotic E. coli fragment, proved to be avirulent.

4. In preliminary experiments, a virulent Salmonella typhimurium strain, C-5, was mated with an avirulent Salmonella abony donor strain. Testing of several hybrid classes produced by this mating indicated that mouse virulence may be lost in the hybrids when substitution occurs in the xyl^+ - rha^+ region.

5. Lactose positive Salmonella typhosa hybrids produced by mating with E. coli Hfr W1895 showed significant increases in frequency when remated with

this Hfr. However, lactose negative segregants from *lac*$^+$ *S. typhosa* heterogenotes displayed no fertility increase, indicating that the integrated *Escherichia* genetic material was responsible for the enhanced recipient ability.

6. *E. coli* Hfr strains W1895 and Hayes were mated with *S. typhosa* 643WS^R with single marker selection for *lac*$^+$ and *ara*$^+$. Four hybrid classes were obtained, each possessing a single marker derived from one of the *E. coli* parents. In a series of eight reciprocal genetic crosses, each hybrid class was remated with each of the two *E. coli* Hfr strains. With one exception, recipient ability was increased in the hybrids only when the *E. coli* genetic segment previously integrated matched the proximal region of the remating Hfr chromosome. We have interpreted this as establishment in *S. typhosa* of an artificial chromosomal homology, which removes the initial barrier to integration of *E. coli* lead markers, thereby increasing the integration chances of more distal Hfr markers.

Discussion

The genetic studies presented in this report confirm and extend the observations of Luria and Burrous (1957) that *E. coli* K-12 and *Shigella* exhibit genetic homology. Our finding that *Shigella* hybrids show alterations in virulence as a consequence of integration of segments of the *Escherichia* chromosome is most significant. Of equal importance is the fact that some hybrids retain virulence even after incorporation of up to 15 per cent of the *Escherichia* genome.

The intermediate virulence of these hybrids which had received one of two widely divergent chromosomal regions is not immediately apparent. However, it

is encouraging that analysis of over one-half of the chromosome has revealed only one region which appears to be essential for the virulence of S. flexneri 2a for the guinea pig. The segregation of virulence among the xyl$^+$ and xyl$^+$ - mal$^+$ hybrids, and the complete loss of virulence in all hybrids carrying the closely linked ind$^+$ and rha$^+$ - ind$^+$ markers indicates that we may be dealing with a single gene, or gene cluster, located between rha$^+$ and xyl$^+$.

The results obtained with partial diploid strains are strongly suggestive that the observed virulence alterations of Shigella hybrids are the direct result of the presence of Escherichia genetic material, rather than some non-specific phenomenon. In the partial diploid, the hybrid retains its full complement of Shigella genes, but is still significantly less virulent than the parental dysentery culture. The strain may return to complete virulence by eliminating the Escherichia chromosomal fragment or become avirulent by incorporation of the fragment. The reduced virulence of the diploid cells indicates that one or more determinants of the endogenote important for virulence are recessive to, or may be replaced by, determinants on the Escherichia exogenote. In addition, the data obtained with diploids suggest that the virulence loss associated with the incorporation of the rha$^+$ - xyl$^+$ region is very likely not due to deletion or unequal crossing over of Shigella material.

As indicated previously, evidence is accumulating to show that the chromosomes of E. coli, Salmonella, and Shigella are grossly homologous. This gross homology could indicate that identical chromosomal regions responsible for virulence (or lack of virulence) may be identified in all three groups of organisms. In fact, our preliminary data with S. typhimurium indicates that this is the case. Further studies will, of course, be required to verify these

data, and to establish the precise chromosomal location of the genetic determinant (or determinants) responsible for virulence in these organisms. In addition, it is anticipated that the information derived from our studies on Salmonella recipient ability will enable us to obtain virulent Salmonella and Shigella donor strains. The employment of these strains in future studies would provide significant information with regard to virulence, genetic homology, and evolution of species within the Enterobacteriaceae.

Literature Cited

Baron, L. S., W. F. Carey, and W. M. Spilman. 1958. Hybridization of Salmonella species by mating with Escherichia coli. Proc. Intern. Congr. Microbiol., 7th Stockholm, p. 50.

Falkow, Stanley, R. Rownd, and L. S. Baron. 1962. Genetic homology between Escherichia coli K-12 and Salmonella. J. Bacteriol. 84: 1303-1312.

Formal, S. B., G. J. Dammin, E. H. LaBrec, and H. Schneider. 1958. Experimental Shigella infections. Characteristics of a fatal infection produced in guinea pigs. J. Bacteriol. 75: 604-610.

Formal, S. B., G. J. Dammin, H. Schneider, and E. H. LaBrec. 1959. Experimental Shigella infections II. Characteristics of a fatal enteric infection in guinea pigs following the subcutaneous inoculation of carbon tetrachloride. J. Bacteriol. 78: 800-804.

Luria, S. E., and J. W. Burrous. 1957. Hybridization between Escherichia coli and Shigella. J. Bacteriol. 74: 461-476.

Zinder, N. D. 1960. Hybrids of Escherichia and Salmonella. Science 131: 813-815.

Bibliography

Baron, L. S., I. Ryman, S. Falkow, and V. Krishnapillai. 1963. Genetic recombination between species of Salmonella. Bacteriol. Proc.

Johnson, E. M., Stanley Falkow, and L. S. Baron. 1963. Recipient ability of Salmonella typhosa in genetic crosses with Escherichia coli K-12. Bacteriol. Proc.

Table 1

Transfer of Genetic Characters from *E. coli* K-12 to *S. flexneri* 2a strain 2457T

Cross	Selection	Frequency of Recombination
E. coli Hfr W 1895	*lac*$^+$	1 X 10^{-3}
X	*ara*$^+$	5 X 10^{-4}
S. flexneri 2a F- 2457T	*rha*$^+$	3.5 X 10^{-5}
	xyl$^+$	2 X 10^{-5}
	mal$^+$	2 X 10^{-5}
	fuc$^+$	5 X 10^{-6}
	nic$^+$	$< 10^{-7}$
E. coli Hfr AB 313	*xyl*$^+$	3 X 10^{-3}
X	*mal*$^+$	1 X 10^{-3}
S. flexneri 2a F- 2457T	*nic*$^+$	5 X 10^{-6}
E. coli Hfr 1362	*mal*$^+$	2 X 10^{-3}
X	*xyl*$^+$	1 X 10^{-3}
S. flexneri 2a F- 2457T	*lac*$^+$	7 X 10^{-5}

The frequency of recombination is expressed as the number of hybrids isolated per donor cell.

Table 2

Frequency of Unselected Markers Among Recombinant Classes Selected for a Distal Marker

Cross	Selection	No. Examined	Per Cent								
			lac$^+$	*ara*$^+$	*pil*$^+$	*rha*$^+$	*ind*$^+$	*xyl*$^+$	*mal*$^+$	*fuc*$^+$	*nic*$^+$
E. coli W1895 × *S. flexneri* 2457T	*mal*$^+$	180	3	1	0	9	10	14	--	2	0
E. coli W1895 × *E. coli* K-12F-	*mal*$^+$	100	56	59	48	N.D.	N.D.	78	--	N.D.	N.D.
E. coli 1362 × *S. flexneri* 2457T	*lac*$^+$	100	--	12	7	1	1	0	0	0	0
E. coli 1362 × *E. coli* K-12F-	*lac*$^+$	100	--	76	N.D.	N D.	N.D.	65	69	N.D.	N.D.

Table 3

Summary of Hybrid Virulence

Hybrid Class	No. hybrid strains tested	Deaths Total	% Mortality	95% Confidence Limits
lac$^+$	3	19/24	79.16	62.90-95.42
lac$^+$ - *ara*$^+$	2	61/77	79.22	70.16-88.28
ara$^+$ - *pil*$^+$	3	16/55	29.09	17.10-49.08
rha$^+$	2	18/24	75.00	57.95-92.05
xyl$^+$	3	20/25	80.00	64.32-95.68
xyl$^+$	3	1/36	2.77	-2.59-8.13
xyl$^+$ - *mal*$^+$	4	39/60	65.00	52.93-77.07
xyl$^+$ - *mal*$^+$	2	2/39	5.12	-1.79-12.03
mal$^+$	5	62/92	67.39	57.81-76.97
ind$^+$ - *xyl*$^+$ - *mal*$^+$	3	4/56	7.14	0.42-13.86
rha$^+$-*ind*$^+$-*xyl*$^+$-*mal*$^+$	4	9/103	8.74	3.54-13.95
fuc$^+$	1	17/25	68.00	49.78-86.82
fuc$^+$	3	11/33	33.33	17.26-49.40
nic$^+$ - *fuc*$^+$	2	6/20	30.00	10.02-49.99
nic$^+$ - *fuc*$^+$ - *lac*$^+$	1	8/21	38.09	18.30-57.88
Controls				
S. flexneri 2457T	1	120/157	76.43	69.79-83.07
E. coli W1895	1	1/48	2.08	-1.96-6.12

Table 4

Virulence of Partial Diploid Hybrids
Of S. flexneri 2a and Their Haploid Segregants
For the Guinea Pig

Genotype of Hybrid	Deaths / Total	% Mortality	95% Confidence Limits
Diploid rha^{+}-ind^{+}-xyl^{+}-mal^{+} $rha^{\blacklozenge}$-$ind^{\blacklozenge}$-$xyl^{\blacklozenge}$-$mal^{\blacklozenge}$	11/29	34.48	19.55-49.61
Haploid Segregant rha^{-}-ind^{-}-xyl^{-}-mal^{-}	24/29	82.76	63.54-92.82
Haploid Segregant rha^{+}-ind^{+}-xyl^{+}-mal^{+}	2/26	7.69	-2.60-17.98

Table 5

Summary of Matings of *S. typhosa* Hybrids with Hfr W1895 and Hfr Hayes

Cross	Selection	Mean Freq./ Donor Cell	Std. Dev.	Control Cross Freq.	Increase Over Control
(1) W1895 x WS^r *lac*$^+$(95)	arabinose	2.0×10^{-4}	±1.2	1.0×10^{-6}	200X
(2) W1895 x WS^r *ara*$^+$(95)	lactose	2.1×10^{-5}	±1.8	1.5×10^{-5}	---
(3) Hayes x WS^r *ara*$^+$(95)	lactose	1.6×10^{-4}	±2.5	6.0×10^{-7}	265X
(4) Hayes x WS^r *lac*$^+$(95)	arabinose	5.5×10^{-6}	±1.5	4.8×10^{-6}	---
(5) Hayes x WS^r *ara*$^+$(H)	lactose	4.7×10^{-5}	±3.2	5.0×10^{-7}	94X
(6) Hayes x WS^r *lac*$^+$(H)	arabinose	8.0×10^{-5}	±3.5	6.0×10^{-6}	13X
(7) W1895 x WS^r *lac*$^+$(H)	arabinose	4.5×10^{-5}	±2.0	8.2×10^{-7}	55X
(8) W1895 x WS^r *ara*$^+$(H)	lactose	1.8×10^{-5}	±1.5	1.5×10^{-5}	---

The frequency of the control cross (W1895 or Hayes x 643WS^r for the selected marker) represents the highest observed recombination frequency of 3 plates set up concurrently with each of the test crosses.

KEY WORDS DEFINING SUBJECT MATTER FOR INDEXING PURPOSES

1. Enteric bacteria

   - genetics of virulence in

2. Genetics

   - of virulence in enteric bacteria

3. Virulence

   - genetic studies in enteric bacteria